Expression of Apoptosis-Related Genes and Proteins in Experimental Chronic Renal Scarring

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Abstract. Apoptosis has been proposed to play an important role in the progression of renal scarring. The mechanisms that determine whether a cell enters the apoptotic program are complex. Bax and Bcl-2 are recognized modulators of this event; their relative levels determine the fate of cells. A role for apoptosis in the progression of renal scarring in the remnant kidneys of rats submitted to subtotal nephrectomy (SNx) has been described. This study investigated the expression (protein and mRNA) of Bax and Bcl-2 in remnant kidneys between day 7 and day 120 post-SNx. Northern blot analysis showed that bax mRNA was increased in remnant kidneys from day 7 and day 120 post-SNx. Northern blot analysis showed that bax mRNA was increased in remnant kidneys from day 7 (150% of control; \( P < 0.05 \)), whereas bcl-2 mRNA was decreased from day 15 (23% of control; \( P < 0.05 \)) resulting in a 14-fold increase in the ratio of bax to bcl-2 mRNA by day 120. Western blot analysis showed similar changes in Bax and Bcl-2 protein in remnant kidneys, resulting in a 147-fold increase in the ratio of Bax to Bcl-2 on day 120. Immunohistochemistry showed increases in Bax to be located predominantly in tubules in SNx kidneys. Interestingly, Bcl-2 immunostaining increased in some epithelial cells within atrophic tubules despite the overall decrease in Bcl-2 protein and mRNA. The overall renal apoptotic cells correlated closely with the ratio of bax to bcl-2 at both the mRNA and protein levels (\( r = 0.594 \) and 0.308, respectively; \( P < 0.05 \)). Furthermore, tubular apoptosis correlated positively with the mRNA level of bax (\( r = 0.471 \); \( P < 0.01 \)) and negatively with the mRNA and protein levels of bcl-2 (\( r = -0.443 \) and \( -0.607 \), respectively; \( P < 0.01 \)). The increase in the ratio of the death inducer (Bax) to the death repressor (Bcl-2) at the mRNA and protein levels may control the apoptosis associated with the progression of tubular atrophy and chronic renal fibrosis within remnant rat kidneys. These observations may have prognostic and therapeutic implications in chronic renal failure.

In chronic nephropathies, the relentless progression of renal scarring results in irreversible chronic renal insufficiency. The progression of chronic renal failure (CRF) is characterized by the loss of renal cells and their replacement by extracellular matrix and fibroblasts. Although a number of studies have focused on mechanisms of renal scarring, the mediators involved remain to be fully elucidated (1). Recently, apoptosis has been implicated with the progressive loss of renal cells during the course of experimental CRF (2–4). Previously, we suggested an important role for apoptosis in the progression of tubular atrophy and CRF in the kidneys of rats submitted to subtotal nephrectomy (SNx). In these rats, cellular apoptosis outweighed proliferation, thus favoring cell deletion and progressive tubular atrophy (5).

An increasing number of genes and their protein products have been associated with the regulation of apoptosis (6,7).

Members of the Bcl-2 family modulate apoptosis in many systems; the ratio of Bax to Bcl-2 determines the fate of cells (8–11). Bcl-2 (B-cell lymphoma/leukemia 2) proto-oncogene has been shown to inhibit apoptosis and prolong cell survival in various settings (9,10). Bax, a proapoptotic antagonist of Bcl-2, has been characterized as a Bcl-2–binding protein that shares significant sequence homology with Bcl-2 (8). Bax and Bcl-2 can either homo- or heterodimerize. Heterodimerization between proapoptotic Bax and antiapoptotic Bcl-2 may negate the function of either protein. An excess of Bcl-2 homodimers favors cell survival, whereas that of Bax homodimers favors cell death (10,11). Thus, the ratio of Bax to Bcl-2 serves as a rheostat to determine the susceptibility to apoptosis (8,12).

Aberrations in the Bcl-2 family result in disordered homeostasis, a pathogenic event in disease (12). Bcl-2 mutation has been implicated in the pathogenesis of polycystic kidney disease in mice (13), and overexpression of Bcl-2 in B lymphocytes of transgenic mice is associated with autoimmunity and proliferative glomerulonephritis (14). Similarly, Bax-deficient mice display cellular hyperplasia (12). The changes of Bax and Bcl-2 expression have been described in various experimental renal models (15,16) and human glomerulonephritis (17). Furthermore, the changes in the Bax to Bcl-2 ratio also have been studied in experimental ischemic renal injury (6) and diabetic nephropathy (18). However, there are limited...
data concerning the relevance of such regulatory pathways to the process of chronic renal cell atrophy and renal fibrosis.

To elucidate the association of apoptosis and its regulating genes and proteins with the progression of experimental CRF, we investigated the expression of Bax and Bcl-2 at the mRNA and protein levels in the rat SNx model of renal scarring. We then correlated these changes to both the progression of renal scarring and the changes in renal apoptosis.

**Materials and Methods**

**Experimental Animals and Protocol**

Male Wistar rats (Sheffield University strain) weighing 300 to 400 g were subjected to subtotal (5/6) nephrectomy (SNx). Rats were housed at constant temperature (20°C) and humidity (45%) on a 12-h light/dark cycle. They were fed ad libitum on standard laboratory rat chow (Lab Sure Ltd., March, Cambridge, UK) and had free access to tap water. SNx was undertaken in 32 rats as a one-step procedure: left 2/3 nephrectomy through the ligation and ablation of kidney upper and lower poles as well as a right uninephrectomy (19). Rats were killed in groups (n = 4 to 6) at days 7, 15, 30, 60, 90, and 120 after SNx. Sham-operated rats (n = 29) were used as controls and were killed at the same time points as those that underwent SNx. Rats were housed in metabolic cages for 24 h before they were killed to facilitate urine collection. All of the experiments were carried out according to the rules and regulations laid down by the Home Office (Animal Scientific Procedure Act 1986, UK).

Removed kidney tissue was fixed in formal calcium (4% wt/vol) paraformaldehyde and 2% (wt/vol) calcium chloride, pH 7.4) and paraffin embedded for histologic and immunohistochemical examination. For electron microscopy, small tissue blocks were fixed in 2.5% (vol/vol) glutaraldehyde solution in phosphate-buffered saline (PBS; pH 7.4). Snap-frozen tissues were stored in liquid nitrogen for protein and mRNA analyses. Serum creatinine concentration (standard auto-analyzer techniques) and 24-h urinary protein excretion (Biuret analyzer techniques) and 24-h urinary protein excretion (Biuret) and mRNA analyses. Serum creatinine concentration (standard auto-analyzer techniques) and 24-h urinary protein excretion (Biuret) and mRNA analyses. Serum creatinine concentration (standard auto-analyzer techniques) and 24-h urinary protein excretion (Biuret) and mRNA analyses. Serum creatinine concentration (standard auto-analyzer techniques) and 24-h urinary protein excretion (Biuret).

**Estimation of Renal Scarring**

For estimation of the severity of renal scarring, Masson’s trichrome stained sections were examined (×200 magnification) and scored by two authors (G.L.T. and A.M.E.N.), who were blinded to the experimental code, according to a previously published arbitrary scale (19–21). Briefly, normal glomerulus scored 0, mild segmental glomerulosclerosis (GS) affecting up to 25% of the glomerular tuft scored 1, moderate GS affecting between 25 and 50% of the tuft scored 2, and severe GS affecting more than 50% of the tuft scored 3. A minimum of 30 glomeruli were scored for each animal and the mean score attributed to the animal.

Similarly, normal tubulointerstitium (tubular cell number approximately 1000/field at ×200 magnification) scored 0, mild tubular atrophy (TA) with interstitial edema or fibrosis (IF) affecting up to 25% of the field (tubular cell number approximately 800) scored 1, moderate TA and IF affecting 25 to 50% of the field (tubular cell number approximately 600) scored 2, and severe TA and IF exceeding 50% of the field (tubular cell number approximately 400) scored 3. The data were collected from a minimum of 12 randomly selected cortical fields and the mean score attributed to the animal. Overall renal scarring was determined using the average of glomerular and tubulointerstitial scores.

**In Situ End Labeling for the Detection of Apoptotic Cells**

In formal calcium-fixed, paraffin-embedded 4-μm sections, fragmented nuclear DNA associated with apoptosis was labeled in situ with digoxigenin-deoxyuridine (dUTP) by terminal deoxynucleotidyl transferase (TdT) (22), using the ApopTag™ Plus peroxidase kit (Appligene Oncor, Illkirch, France) as per the manufacturer’s instructions. Briefly, after deparaffinization and hydration, sections were digested by incubation with 15 μg/ml proteinase K for 15 min at 37°C to enable penetration of TdT into the section for even incorporation of nucleotides. Endogenous peroxidase was inactivated by 2% (vol/vol) H2O2 in PBS. The sections were then immersed in TdT reaction buffer and incubated with TdT and digoxigenin-dUTP for 60 min at 37°C. The slides were transferred to stop buffer at 37°C for 30 min to terminate the reaction. The sections were incubated with the anti-digoxigenin-peroxidase complex for 30 min at 37°C and developed by using the 3’-amino-9-ethylcarbazole substrate kit (Vector Laboratories, Peterborough, UK) and counterstained with hematoxylin. For negative controls, slides were incubated in TdT buffer without TdT. For biochemically induced positive controls, slides were pretreated with 10 μg/ml DNase I (Sigma, Dorset, UK) in DNA buffer.

For each experimental animal, more than 30 glomerular cross sections and 25 high power (×400 magnification) fields of tubulointerstitium were examined by two authors (B.Y. and G.L.T.) who were blinded to the experimental code. The number of in situ end labeling (ISEL) positive-staining nuclei per glomerulus, per 400 tubular cells, or per interstitial field determined the score of glomerular (Gapo), tubular (Tapo), or interstitial (lapo) apoptosis, respectively. ISEL of DNA while associated with apoptosis can also be seen in necrotic (nonspecific DNA degradation) and mitotic (transient DNA strand break) cells. To substantiate the specificity of our results, we confirmed apoptosis by light microscopic evaluation of the characteristic morphologic features; only strongly positive ISEL cells with observable morphologic features of apoptosis such as shrunken cells with condensed nuclei surrounded by a narrow cytoplasmic halo were counted (22,23,24).

**Northern Blot Analysis of Bax and Bcl-2 mRNA**

Total RNA was extracted using TRizol reagent (Life Technologies BRL, Paisley, UK) and quantified by scanning spectrophotometer at 260 nm. Fifteen μg of total RNA was electrophoresed on a 1.2% (wt/vol) agarose MOPS/formaldehyde gel and viewed under ultraviolet (UV) light to verify loading and the presence of intact ribosomal bands. RNA was then transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK) by capillary blotting and fixed by UV cross-linking (UV cross-linker, Amersham Pharmacia Biotech Ltd.) at 70 mICm² (25).

To obtain bax and bcl-2 cDNA probes, sequences were amplified from rat cDNA by the PCR. Bax was amplified using previously published primers: 5’sense CCTAGGACGTCTGAGACATGC, 3’antisense CTCTTTCCAGATGTTGAC (18). Bcl-2 primers (5’sense CTTTAGAGCAACCCGAAAC, 3’antisense CCTGAGAGCAACCGAACC) were designed based on a rat cDNA sequence (Genebank accession number, RNBCL2A) (26). Amplification reactions were performed with 20 pmol of primer, 100 μM of each dNTP, amplification buffer (containing 1.5 mM MgCl2), and 1 unit of Taq polymerase. The 39 cycles of amplification were completed using the following condition: 94°C for 1 min, 55°C (bac) or 48°C (bcl-2) for 1 min, 72°C for 1 min. The 500-bp (bax) and 450-bp (bcl-2) PCR products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Recombinant clones were selected and amplified and plasmid
DNA was harvested using the Wizard Maxiprep Kits (Promega, Southampton, UK). The bax and bcl-2 inserts were released with an EcoRI digestion, separated by agarose electrophoresis and purified using the Prep-A-Gene DNA Purification Systems (Bio-Rad Laboratories Ltd., Harfordshire, UK). Product confirmation was by restriction mapping using EcoO109I and Ddel for bax; BstXI and Ddel for bcl-2 (Appligene Oncor). Purified cDNA were random primed with 32P-labeled dCTP (NEN, Boston, MA) using the Prime-a-Gene Labelling System (Promega). Purified cDNA were random primed with 32P-labeled dCTP (NEN, Boston, MA) using the Prime-a-Gene Labelling System (Promega). Unincorporated label was removed using Sephadex G-50 NICK™ Column (Pharmacia Biotech, Herts, UK).

Prehybridization and hybridization were performed as described in detail elsewhere (25). Briefly, filters were prehybridized in a mixture containing 50% (vol/vol) denatured formalinized, 5× salt sodium phosphate ethylenediaminetetraacetic acid (SSPE), 5× Denhardt’s reagent, 1% (wt/vol) sodium dodecyl sulfate (SDS), and 200 µg/ml denatured herring sperm DNA, at 42°C for at least 1 h. Hybridization was performed under the same conditions with the addition of labeled probes overnight. Membranes were washed twice using 0.2% SDS/2×SSPE at room temperature and then twice in 0.2% SDS/0.2×SSPE at 65°C before being exposed to Kodak Biomax MS film (Sigma). Autoradiographs were quantitatively analyzed using a Bio-Rad GS-690 scanning densitometer and Molecular Analyst version 4 software. Volume density values were corrected for loading using the housekeeping gene cyclophilin (27). Results are expressed as percentage of control sample mRNA densities. The bax to bcl-2 mRNA ratio was determined for each sample individually by reprobing of the same membrane and dividing the volume density for bax by that for bcl-2. Data were standardized by dividing the mean Snx ratio by the mean control ratio at each time point.

**Measurement of Tissue Bax and Bcl-2 Protein Levels**

Tissue levels of Bax and Bcl-2 proteins were determined by immunopробing of Western blots. A volume equivalent to 20 µg of protein from a 20% (wt/vol) tissue homogenate was separated on a 15% (wt/vol) polyacrylamide denaturing gel and then electroblotted onto Hybond ECL nitrocellulose (Amersham Pharmacia Biotech Ltd.). The membranes were blocked by the addition of 3% (vol/vol) bovine serum albumin in 0.1% (vol/vol) Tween-20 Tris-buffered saline at 4°C overnight. The Western blots were probed with a polyclonal rabbit anti-rat Bax 1:2000 (P-19, Santa Cruz Biochemicals, Santa Cruz, CA) at room temperature for 2 h. Primary Bax antibody binding was revealed using an anti-rabbit horseradish peroxidase (HRP) at 1:2000 dilution (Dako, Glostrup, Denmark) for 1 h and the ECL chemiluminescent detection system (Amersham Pharmacia Biotech). The membranes were then reprobed with a monoclonal mouse anti-rat Bcl-2 at 1:2000 dilution (Santa Cruz Biochemicals). Bcl-2 antibody binding was revealed using a biotinylated anti-mouse antibody 1:1000 for 1 h followed by a HRP-conjugated avidin (ABC Elite, Vector Laboratories) at a 1:1000 dilution for 1 h before chemiluminescent detection. Developed films were analyzed quantitatively by volume densitometry using a Bio-Rad GS-690 scanning densitometer and Molecular Analyst version 4 software (Bio-Rad Laboratories Ltd.). The Bax to Bcl-2 ratio was determined in the same manner as that for mRNA.

**Double Immunostaining for ISEL, Bax, and Bcl-2, and PCNA and Bcl-2**

Double immunohistochemical staining was undertaken on paraffin sections. For ISEL and Bax or Bcl-2, 4-µm sections were dewaxed and hydrated, then processed as described above in the ApopTag method. Afterward, they were preincubated with blocking serum for 30 min and labeled with anti-Bax or Bcl-2 antibody at 4°C overnight. Sections were then washed in 0.05% (vol/vol) Tween-20/PBS and PBS before addition of a 1:1000 dilution of a donkey anti-rabbit Cy5 conjugated antibody (Stratech Scientific, Luton, UK) and incubation at 37°C for 30 min. Sections were washed and mounted with UV-free aqueous media and dextropropoxyphene media and then stored at −20°C until observation. Sections were visualized using a confocal microscope (Leica DMRBE, Solms, Germany) with a Kr/Ar laser providing excitation at 488 and 647 nm. Emission wavelength measurements were taken at 530 nm and 665 nm for autofluorescence and Cy5, respectively.

**Immunostaining for Bax and Bcl-2 Proteins**

Localization of Bax and Bcl-2 proteins was performed in formal calcium-fixed, paraffin-embedded kidney tissue by immunohistochemistry using a standard avidin-biotin peroxidase complex technique as described previously (20). Four-µm sections were dewaxed and hydrated. After the quenching of the endogenous peroxidase activity by 3% (vol/vol) H2O2 in methanol, sections were incubated with the primary antibody (polyclonal rabbit anti-mouse Bax [P-19] antibody [Santa Cruz Biochemicals]) diluted 1:50 or polyclonal rabbit anti-rat Bcl-2 antibody (Pharmingen, San Diego, CA) diluted 1:400. Confirmatory Bcl-2 staining was with both a monoclonal mouse anti-rat Bcl-2 antibody (Santa Cruz Biochemicals) diluted 1:50 and a rabbit anti-mouse Bcl-2 antibody (Calbiochem, Nottingham, UK) diluted 1:50 overnight at 4°C in a humid atmosphere. Thereafter, the sections were stained by an avidin-biotinylated HRP procedure using a commercially available kit (ABC Elite, Vector Laboratories). 3’-amino-9-ethylcarbazole was used as the substrate. Finally, sections were counterstained with hematoxylin and mounted in Glycergel (Dako). Control sections were incubated with either normal rabbit IgG or nonimmune normal rabbit serum at the same protein concentration as the primary antibody.

For immunofluorescence staining, the primary antibodies were diluted in 5% (vol/vol) donkey serum/PBS at the same dilution as for immunohistochemistry, then incubated at 4°C overnight. Sections were then washed in 0.05% (vol/vol) Tween-20/PBS and PBS before addition of a 1:1000 dilution of a donkey anti-rabbit Cy5 conjugated antibody (Stratech Scientific, Luton, UK) and incubation at 37°C for 30 min. Sections were washed and mounted with UV-free aqueous media and dextropropoxyphene media and then stored at −20°C until observation. Sections were visualized using a confocal microscope (Leica DMRBE, Solms, Germany) with a Kr/Ar laser providing excitation at 488 and 647 nm. Emission wavelength measurements were taken at 530 nm and 665 nm for autofluorescence and Cy5, respectively.

**Statistical Analyses**

Results are expressed as mean ± SEM. The statistical difference was assessed by a single factor ANOVA. Linear regression and correlation analysis was applied to determine both the correlation r values and P values between parameters. A t test was used for analyzing the expression of tissue bax and bcl-2 mRNA and proteins. P < 0.05 was considered to be significant.
Results
Renal Function and Histology Studies
Proteinuria and serum creatinine concentrations in SNx rats were significantly raised from day 7 and day 30 onward, respectively, reaching 256.8 ± 56.8 mg protein/24 h and 140.6 ± 36.5 μmol/L creatinine by day 120 compared with control levels of 7.4 ± 0.8 mg protein/24 h and 40.0 ± 1.4 μmol/L creatinine. This indicated progressive renal insufficiency as previously documented in our earlier studies using this model (5). Significant evidence of GS, TA, and IF after SNx was noted from day 7 onward and progressively increased thereafter. At day 120, the scores of GS, TA (tubular cell number/×200 field), and IF were 1.86 ± 0.15, 613 ± 47, and 1.93 ± 0.24, respectively. In the rats subjected to SNx, the overall renal scarring was moderate in severity (scarring index of 1.9 ± 0.2) by the end of the experiment.

Detection of Apoptosis
With the use of ISEL, very few apoptotic cells were noted in the glomeruli, tubules, and interstitium of sham-operated rats. The remnant kidneys demonstrated a significant gradual increase in positively stained nuclei in the glomeruli from day 15 and in the tubules and interstitium from day 7 until the end of the time course (Table 1). Maximal areas of apoptotic cells were detected in the sclerotic glomeruli (Figure 1A), dilated or atrophied tubules (Figure 1B), and expanded interstitium (Figure 1C). In positive control sections treated with DNAse I before the TdT reaction, nearly all of the cells stained, but most of positive nuclei showed normal shape and no cytoplasmic condensation (not shown). No staining was present in the negative control sections using buffer lacking TdT (not shown). Electron microscopy confirmed apoptotic cells with distinct morphologic motifs (Figure 1, D through F).

Expression of Bax and Bcl-2 Genes
Northern blotting revealed the presence of two bax mRNA transcripts (a predominant 1.0 kb species and a less abundant 1.5 kb species) and the presence of a bcl-2 transcript of 7.5 kb (Figure 2). In comparison with sham-operated rat kidneys, the level of bax mRNA was markedly increased from day 7 (150%; P < 0.05) onward, reaching a peak on day 120 (224%; P < 0.01). By contrast, the expression of bcl-2 mRNA was significantly decreased in remnant kidneys from day 15 (24%; P < 0.05) and remained lower than controls throughout the experimental period (Figure 2). Therefore, the ratio of bax to bcl-2 mRNA was increased throughout the time course and peaked at day 120 with a 14-fold increase in the ratio of bax to bcl-2 (Figure 3A).

Table 1. The time course changes of cell apoptosis in progressive renal scarring

<table>
<thead>
<tr>
<th>Days</th>
<th>Gapo (SNc)</th>
<th>SNx</th>
<th>Tapo (SNc)</th>
<th>SNx</th>
<th>Iapo (SNc)</th>
<th>SNx</th>
</tr>
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<tbody>
<tr>
<td>7</td>
<td>0.017 ± 0.007</td>
<td>0.056 ± 0.016</td>
<td>0.086 ± 0.012</td>
<td>1.077 ± 0.239</td>
<td>0.174 ± 0.020</td>
<td>0.428 ± 0.050</td>
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<tr>
<td>15</td>
<td>0.013 ± 0.007</td>
<td>0.067 ± 0.017</td>
<td>0.092 ± 0.014</td>
<td>0.919 ± 0.080</td>
<td>0.140 ± 0.037</td>
<td>0.457 ± 0.061</td>
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<tr>
<td>30</td>
<td>0.033 ± 0.011</td>
<td>0.117 ± 0.025</td>
<td>0.058 ± 0.015</td>
<td>1.087 ± 0.220</td>
<td>0.178 ± 0.024</td>
<td>0.734 ± 0.098</td>
</tr>
<tr>
<td>60</td>
<td>0.017 ± 0.010</td>
<td>0.100 ± 0.011</td>
<td>0.096 ± 0.010</td>
<td>1.663 ± 0.137</td>
<td>0.178 ± 0.030</td>
<td>0.847 ± 0.118</td>
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<td>90</td>
<td>0.020 ± 0.013</td>
<td>0.207 ± 0.036</td>
<td>0.080 ± 0.012</td>
<td>2.090 ± 0.531</td>
<td>0.206 ± 0.038</td>
<td>0.961 ± 0.308</td>
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<tr>
<td>120</td>
<td>0.033 ± 0.015</td>
<td>0.253 ± 0.040</td>
<td>0.057 ± 0.019</td>
<td>2.766 ± 0.439</td>
<td>0.109 ± 0.032</td>
<td>1.039 ± 0.246</td>
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*a* Gapo, apoptotic cells per glomerulus; Tapo, apoptotic cells per 400 tubular cells; Iapo, apoptotic cells per 400 magnification field. Data represent mean ± SEM. Sham operated (SNc; n = 4) and subtotally nephrectomized (SNx; n = 6).

b P < 0.05.

P < 0.01.
which was mainly but not exclusively confined to the atrophic tubules (dilated lumen, flattened epithelial cells often surrounded by inflamed cells, Figure 6B). No staining was observed in sections that were incubated with normal IgG or serum (Figure 5E and Figure 6C). The pattern of Bcl-2 immunostaining was consistent among all three antibodies used;
however, the Pharmingen polyclonal rabbit anti-rat Bcl-2 antibody gave the most intense staining.

The pattern of immunofluorescence staining for Bax and Bcl-2 was consistent with that of immunohistochemistry. Immunofluorescence staining of Bax was visibly increased in damaged tubules (Figure 7, A1 and A2) and glomeruli (Figure 7, B1 and B2), whereas Bcl-2 immunofluorescence was visible only in a small number of atrophic tubular cells (Figure 7, C1 and C2).

**Double Staining for Apoptosis, Bcl-2, and Bax**

Typically, ISEL-positive cells stained for Bax (Figure 5F); however, some cells stained positively for both apoptosis (ISEL) and Bcl-2 (Figure 6D). Some cells that double-stained for apoptosis and Bax had sloughed into the tubular lumen (Figure 5F). A number of cells, including some tubular cells, stained positively for both Bax and Bcl-2 (Figure 6E). PCNA and Bcl-2 double staining positive cells were also found in some tubules (Figure 6F).

**Correlation among Renal Function and Histology Parameters, Apoptosis, Bax, and Bcl-2 Measurements**

Proteinuria (UP) and serum creatinine (SCr) showed a close correlation with parameters of renal scarring. The number of apoptotic cells in the glomeruli, tubules, and interstitium compartments correlated significantly with the severity of glomerulosclerosis, tubular atrophy, and interstitial fibrosis, respectively (Table 2).

The score of apoptotic cells in the glomeruli, tubules, and interstitium correlated positively with bax mRNA but not Bax protein and correlated negatively with bcl-2 mRNA and protein. The apoptotic index in individual renal compartments (particularly in tubules) correlated positively with the ratio of...
bax to bcl-2 at both mRNA and protein levels (Table 2). The overall renal apoptosis closely correlated with the ratio of bax to bcl-2 mRNA and protein (Figure 8).

**Discussion**

Apoptosis plays an essential role in the regulation of renal cell number in both healthy and diseased kidneys (28). Efficient deletion by apoptosis of excessive, damaged, or nonfunctioning renal cells and infiltrating inflammatory cells may be beneficial (28). This has been suggested in the anti–thy-1.1 model of glomerulonephritis (29). During the progression of experimental crescentic glomerulonephritis to end-stage renal failure, apoptosis also seems to play an essential role in the resolution of intra- and extraglomerular inflammation (2). However, the loss of resident renal cells by uncontrolled apoptosis is detrimental as it may induce a reduction of functional renal mass and lead to renal insufficiency (2).

We previously described a progressive and sustained increase in the number of apoptotic cells in the glomeruli, tubules, and interstitium of remnant rat kidneys, with maximal areas of apoptosis detected in sclerotic glomeruli, atrophied tubules, and expanded interstitium (5). There was a close correlation between the number of apoptotic cells and the parameters of renal scarring, indicating that apoptosis of renal cells might contribute to the progression of tubular atrophy and chronic renal fibrosis (5). Having reproduced these findings in the current study, we aimed to identify the involvement of some regulators in the process of apoptosis. For that, we studied the expression of Bax (proapoptotic antigen) and Bcl-2 (antiapoptotic antigen) at both the mRNA and protein levels.

We noted that both bax transcription and tissue protein levels were significantly increased during the progression of renal scarring. This was localized by immunohistochemistry to damaged glomeruli, tubules, and interstitial cells in remnant kidneys. The detectable expression of Bax was more widespread than that of Bcl-2, which is consistent with the observations made by others in mice (30). As previously reported (6), we detected a low expression of Bax protein in the distal tubules, cortical collecting ducts, and medullary loops of Henle in the kidneys of sham-operated rats. In remnant kidneys, immunostainable Bax was increased in the tubules, especially in dilated tubules, as well as in some sclerotic glomeruli and fibrotic interstitial areas. Elevated Bax protein has also been observed in models of renal ischemia/reperfusion (6), where the level of renal bax mRNA is elevated after ischemia and both bax mRNA and protein are co-localized in the regenerating proximal tubules. Others have also reported that Bax-positive glomerular cells were increased with time after SNx and correlated this with apoptosis (15).

In normal kidneys, Northern and Western blot analyses
Figure 5. Distribution of Bax in day 90 sham-operated and remnant kidneys. Pictographs of sham-operated kidneys (×100) show Bax immunostain in the distal tubules (A), cortical collecting ducts (A), and Henle’s loop of medulla (B). In remnant kidneys, Bax immunostaining is seen in the dilated proximal tubular cells (C), damaged glomerulus (D), and interstitial cells (D). (E) A nonimmune control on a remnant kidney. (F) Co-localization of Bax (bright pink) and in situ end labeling (ISEL; apoptosis, brown) in tubular cells of remnant kidney. Magnifications: ×100 in A and B; ×400 in C and F; ×200 in D and E.
Figure 6. Immunostain for Bcl-2 in day 90 sham-operated and remnant kidneys. Pictographs show Bcl-2 immunostain in a sham-operated kidney (A) and atrophied tubules of remnant kidneys (B). (C) A nonimmune control in a remnant kidney. Double staining of Bcl-2 (bright pink) with ISEL (brown) in atrophied tubular cells of remnant kidneys (D), dark brown indicating composite staining. Double staining of Bax (bright pink) and Bcl-2 (brown) in the dilated and atrophied tubular cells of remnant kidney (E). Double staining of Bcl-2 (bright pink) and proliferating cell nuclear antigen (PCNA; brown) in the atrophied tubular cells of remnant kidney (F). Magnifications: ×200 in A and C; ×400 in B, D, E, and F.
Figure 7. Distribution of Bax and Bcl-2 in the remnant kidneys by confocal microscopy. Damaged tubules (A) and glomerulus (B) are stained for Bax. Atrophied tubules (C) are stained for Bcl-2. 1 denotes combined emissions for positive staining and autofluorescence (emission at 665 and 530 nm); 2 indicates positive staining only (emission at 665 nm).
show abundant levels of bcl-2 mRNA and protein; however, immunostaining shows no or very faint positive pattern. To confirm Bcl-2 staining, we used three different commercial antibodies and two staining techniques and obtained very similar patterns and levels of staining. This may be because each cell expresses a small amount of constitutive Bcl-2, such as in techniques in which total tissue bcl-2 mRNA or protein is concentrated in a single band and detection is possible. However, with the use of immunostaining, the level per cell may be too low to reach the threshold of detection. Predictably, during progressive renal scarring, the total Bcl-2 mRNA and protein levels in the tissue and hence most cells falls. In contrast, a small number of cells (mainly within atrophic tubules) seem to upregulate Bcl-2, probably in an attempt to prevent further cell deletion by apoptosis. This pattern of raised immunostainable and inducible Bcl-2 in some tubular cells with an overall decreased level in the remnant kidney suggests a greater reduction of the Bcl-2 content in other renal cells that may contribute toward the induction of apoptosis in these cells of remnant kidneys. Of interest, some cells, including tubular cells, showed double staining for Bax and Bcl-2. The co-localization of Bax and Bcl-2 has previously been described in cells showing different degrees of DNA fragmentation within the small intestine and the kidney (31). The localization of both Bax and Bcl-2 in the same cells may indicate that two opposing mechanisms are trying to determine the fate of the cells or an ancillary role for Bcl-2 independent of its apoptotic regulatory function.

The localized rise in Bcl-2 protein in some remnant tubules seems in contradiction to its apoptotic regulative function and its tissue mRNA and protein expression. There may be several possible explanations for this. First, the increased immunostainable Bcl-2 in damaged tubules may be a protective mecha-

| Table 2. Correlation among renal function, histology, apoptosis, Bax, and Bcl-2.
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Figure 8. Correlation between overall renal apoptosis score and the ratio of bax and bcl-2 at the mRNA and protein levels in control and SNx rat kidneys.
anism that prevents further cell loss by providing increased time for DNA repair to occur before succumbing to apoptosis (24). Second, the precise function of the members of the Bcl-2 family remains somewhat uncertain. Bcl-2 is a membrane-bound protein that is present in mitochondria, nucleus, and endoplasmic reticulum membranes (32,33), which may have an antioxidant role, thus preventing lipid peroxidation and the associated cellular toxicity (33–36). In the course of the remnant kidney nephropathy, tubular hypermetabolism and hyperoxidation have been put forward as possible initiators of cellular injury and scarring (37,38). The upregulation of Bcl-2 in some of these remnant tubules may therefore have a protective effect against such oxidative stress. Third, Bcl-2 may have been localized in regenerating tubular cells within atrophic tubules. Increased levels of renal bcl-2 mRNA and protein have been described in regenerating tubular cells after an ischemic injury (6). Renal scarring has been associated with progressive renal ischemia and hypoxia (39,40). Therefore, a protective role for Bcl-2 in ischemic and regenerating tubular cells cannot be excluded. This is supported by our observation of Bcl-2 and PCNA double staining positive patterns in some tubules within remnant kidneys. Finally, the Bcl-2 antiapoptotic influence is modulated by other members of its family, including Bcl-xL (41,42), which have not been measured in this study and warrant further investigation.

Because of the interplay between members of the Bcl-2 family, perhaps the most important observation is the changes in the ratio of Bax to Bcl-2. At both the mRNA and protein levels, there is a strong increase in this ratio, which would favor apoptosis (especially in tubules). Interestingly, whereas at the mRNA level this increase is progressive, at the protein level there are distinct early and late peaks that coincide with compensatory renal growth (glomerular and tubular hypertrophy) that occurs immediately after ablation and the late progressive glomerulosclerosis and tubulointerstitial scarring (43). The differences may indicate further translational or posttranslational control of these proteins within the diseased kidney. Furthermore, renal apoptosis correlated positively with the mRNA level of bax and negatively with the mRNA and protein levels of Bcl-2. As Bcl-2 protein shows a stronger negative correlation with renal apoptosis than the positive Bax protein correlation, this may indicate that regulation of Bcl-2 is potentially of greater importance in renal cell deletion than that of Bax.

Although the progression of renal scarring increases the level of apoptosis within the remnant kidney and this seems to some degree to be mediated through the Bax and Bcl-2 system, the factors that alter the kidney to modulate Bax and Bcl-2 remain to be determined. Previous studies have shown changes in a range of growth factors and cytokines within the scarred kidney (18,44), and many of these have been shown to influence apoptosis under certain conditions, e.g., transforming growth factor-β (44,45) and tumor necrosis factor-α (46) inducing apoptosis and insulin-like growth factor-1 (46,47) and fibroblast growth factor (47) suppressing apoptosis. Alternatively, high-protein environments such as those associated with proteinuria have been shown to influence apoptosis both in vitro (48,49) and in vivo (50). Other possible factors include the microischemia that occurs in the tubules during progressive scarring (39,40) given that hypoxic conditions have been shown to be a strong inducer of apoptosis in vitro (51) and ischemic injury is associated with raised apoptosis in the kidney (52). Interestingly, recent studies have suggested that growth factors such as insulin-like growth factor-1 and epidermal growth factor acted in concert with and Bcl-2 to promote renal cell survival in ischemic renal injury (21), which may account for the cell-specific Bcl-2 upregulation seen in this study. It is unlikely that a single factor will be responsible for the changes of Bax and Bcl-2. However, these modulators of apoptosis are potentially attractive targets for the regulation of apoptosis.

We conclude that the apoptosis and its modulators (Bax and Bcl-2) are associated with progressive tubular atrophy, glomerulosclerosis, and interstitial fibrosis in experimental renal scarring in rats that are subjected to extensive renal ablation. This may have therapeutic implication as the manipulation of these regulators may modulate apoptosis and prevent the progressive cellular depletion associated with renal fibrosis and chronic renal insufficiency.

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References


